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Immunoblot Analysis of Exospore Polypeptides from Some Entomophilic Microsporidia

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16. ABSTRACT

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Immunoblot Analysis of Exospore Polypeptides from Some Entomophilic Microsporidia[1,2]

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ABSTRACT. Immunological relationships between genera and species of microsporidia were examined by immunoblot analysis. Exospore polypeptides from two *Nosema* spp., three *Vairimorpha* spp., and two undescribed *Vairimorpha*-like isolates were analyzed. Gel electrophoresis and immunoblot analysis revealed that a variety of polypeptides, mostly between 15,000 and 90,000 molecular weight, are present on the exospore. The three *Vairimorpha* spp. are closely related immunologically to each other, but less so to the two undescribed *Vairimorpha*-like isolates. The two *Nosema* spp. are immunologically distant from each other and from the *Vairimorpha* spp. Indirect evidence, however, indicated that many internal spore polypeptides present in both genera are similar. Cross-reactivity between exospore polypeptides from entomophilic microsporidia and antisera to a mammalian microsporidium, *Encephalitozoon cuniculi*, was very limited. These results indicate that immunoblot analysis of exospore polypeptides may be employed to investigate the interrelatedness of microsporidian species, and that exospore polypeptides of some microsporidia are sufficiently diverse to be of immunodiagnostic value.

THE Microspora comprise a diverse group of parasitic protozoa that have been increasingly studied in recent years, largely due to their potential for use in the biocontrol of insect pests (1). One microsporidian species, *Nosema locustae*, has been approved and registered by the US Environmental Protection Agency (3) for use in residual control of rangeland grasshoppers (10). Several other species, particularly members of the genera *Nosema* and *Vairimorpha*, have also received considerable attention as candidate biocontrol agents. The small (2–10 × 1–4 μm), ovoid spores produced by these species, as well as those produced by members of several other microsporidian genera, are usually difficult to distinguish microscopically, and specific identification is often somewhat subjective, usually involving extensive light and electron microscopic studies (9). The spores, however, are the only stable form of these protozoa available in quantity and are also the form used in biocontrol applications. Consequently, we wanted to investigate further methods of readily distinguishing between the spores of Microspora, particularly entomophilic species.

Several earlier attempts have been made to distinguish between spores of microsporidian species through the use of biochemical and immunological methods. A diverse array of antigenic substances has been demonstrated for a variety of species by immunological techniques, when either disrupted spores (8, 11) or intact spores (14, 19) were employed as immunogens. A wealth of extractable proteins has also been demonstrated by disc gel electrophoresis (4, 5), microelectrophoresis (J. D. Knell, pers. commun.), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21, 22) when polypeptides were obtained by trichloroacetic acid precipitation of disrupted spore homogenates. One study (J. D. Knell, pers. commun.) also indicated that species-specific arrays of polypeptides, as shown by microelectrophoresis, could be obtained from the proteinaceous exospore of intact spores.

Gel electrophoresis and an immunoblot assay (18, see also 7) were utilized in the present study in order to determine more precisely the nature of proteins present on the microsporidian exospore. In particular, we were looking for species-specific polypeptides, with both unique electrophoretic mobility and antigenic determinants, suitable for immunodiagnostic use with high affinity reagents such as monoclonal antibodies.

MATERIALS AND METHODS

Microsporidia. Spores of seven species or isolates of microsporidia were received from several sources (Table I). Spores of all species, except *Nosema locustae*, were propagated in *Heliothis zea* (Lepidoptera: Noctuidae) larvae. Larvae were inoculated by allowing them to feed on the surface of spore-treated, artificial diet. Infected larvae were collected prior to pupation and stock suspensions of spores harvested from them were stored in distilled water (DW) at 4°C.

Purification of spores. Spores were obtained from infected insects by trituration in either a Ten-Broeck type tissue grinder, a Virtis cell homogenizer, or a Sorvall Omnimixer. Diluted homogenates were filtered through cheesecloth and then centrifuged at 3400–9000 *g* for 10 min at 4°C to pellet spores and other particulate matter. The supernatant and fat globules were discarded, and the pellet was resuspended in DW and re-centrifuged. Host debris remaining on the pellet surface was removed with a pasteur pipet.

Spores were purified further by density gradient ultracentrifugation. Five to 8 ml of spore suspensions were loaded onto 30 ml of concave (25–60% NaK tartrate [w/w]) density gradients made with an Instrumentation Specialties Model 570 gradient maker. Concavity in the gradients was produced by mixing the heavy : light components of the gradient at a ratio of 2.75:1, respectively. Spores were centrifuged to quasi-equilibrium at 170,000 *g* for 30 min at 15°C. This usually produced a discrete, whitish, turbid zone which contained most of the mature, intact spores. This spore zone was removed and the spores washed twice with DW. This purification procedure was repeated two or more times with each spore preparation. If any visible contaminants remained, spore suspensions were subjected to rate zonal centrifugation in similar concave gradients of 20–60% sucrose (w/w) at 170,000 *g* for 15 min at 15°C, followed by two washes in DW. Spore suspensions were considered sufficiently purified when the spore to particulate contaminant ratio exceeded 1000:1; however, *N. heliothidis* spores could not be purified to this extent.

Preparation of samples for SDS-PAGE. Samples of exospore polypeptides for SDS-PAGE were obtained by boiling ca. 1 × 10^8 spores for 10 min in 100 μl of an extraction buffer containing 5% beta-mercaptoethanol (w/v), 2% SDS (w/v), and 0.005% bromophenol blue (w/v) in 0.06 M Tris-HCl buffer, pH 6.8. All samples were prepared immediately prior to electrophoresis.

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[2] This paper has been reviewed by the Health Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

TABLE I. *Sources of Microsporidia.*

Microsporidian isolate	Host[a]	Original host[b]	Geographic origin	Source
Nosema heliothidis Lutz & Splendore	*Heliothis zea*	*H. zea*	Georgia	J. J. Hamm, USDA-SEA-AR, Tifton, GA
Nosema locustae Canning	*Melanoplus bivittatus*	*Melanoplus* spp.	Montana	J. E. Henry, Rangeland Insect Lab, USDA-SEA, Montana State Univ., Boseman, MT
Vairimorpha heterosporum (Kellen & Lindegren)	*Spodoptera exigua*	*Plodia interpunctella*	Illinois	J. V. Maddox, Ill. Nat. Hist. Surv., Urbana, IL
Vairimorpha plodiae (Kellen & Lindegren)	*S. exigua*	*P. interpunctella*	Illinois	J. V. Maddox
Vairimorpha necatrix (Kramer)	*H. zea*	*Hyphantria cunea*	Illinois	J. V. Maddox
UBV	*H. zea*	*Utethesia bella*	Florida	J. V. Maddox
78-5	*H. zea*	*Alabama argillacea*	Bolivia	J. J. Hamm

[a] Host in which isolate received was originally grown; propagation (except *N. locustae*) for experimental use was in *H. zea*.
[b] Host in which isolate was originally discovered.

Samples of *H. zea* hemolymph were taken from healthy fifth-instar larvae. Larvae were surface-sterilized by immersion in 95% ethanol for 2 min, followed by a brief rinse in DW. Hemolymph was collected by snipping off a larval proleg and drawing up the exuding fluid with a capillary tube. Hemolymph was emptied into an antioxidant solution containing 50 mM dithiothreitol and 10 mM Tris-HCl buffer, pH 7.3. Sufficient hemolymph was collected to make a 1:1 mixture of antioxidant solution and hemolymph. This was mixed well and centrifuged for 5 min at 15,600 *g*. The supernatant was collected, mixed 1:20 with extraction buffer, and stored at −20°C until needed. Samples were boiled for 10 min immediately prior to electrophoresis.

Samples of *H. zea* fat body were also taken from surface-sterilized, fifth stage larvae after dissection in dithiothreitol anti-oxidant solution. Pieces of fat body were placed in a 1:1 mixture of extraction buffer and anti-oxidant solution, triturated, and the homogenate centrifuged as before. Fat globules were discarded, and the remaining supernatant mixed 1:1 with extraction buffer and stored −20°C until needed. Samples were boiled for 10 min immediately prior to electrophoresis.

Samples of molecular weight marker proteins (LMW Gel Calibration Kit, Pharmacia Fine Chemicals) were prepared by reconstitution of 250 μg of protein in 100 μl of extraction buffer and boiling for 10 min. Samples were prepared fresh immediately prior to electrophoresis.

SDS-PAGE of polypeptide samples. Electrophoresis of polypeptide samples of spores and control fat body and hemolymph was performed on a vertical slab gel apparatus (BioRad) in SDS-polyacrylamide gels essentially as reported (12). Resolving gels, 0.075 cm thick and containing 10% acrylamide and 0.4% SDS, were poured to a depth of 9 cm. Stacking gels of 4% acrylamide were 3 cm deep.

An electrophoresis buffer containing 192 mM glycine, 0.1% SDS (w/v), and 25 mM Tris-HCl, pH 8.3, was employed. Samples (10 μl) were electrophoresed at 20 mA until the bromophenol blue dye front reached the resolving gel (ca. 1 h). Current was increased to 40 mA until the dye front was 1–2 mm from the bottom of the gel (ca. 3 h). Gel temperature was maintained at ca. 20°C by water circulation through the gel apparatus core.

Gels thus prepared were either stained or used to prepare gel blots (see below). Gels were prepared for staining by fixation for 20–30 min in 45% methanol, 45% DW, and 10% glacial acetic acid. Silver staining was then conducted as in ref. 15. A record of a stained gel was obtained by photographing it on a light box.

Preparation of gel blots. After electrophoresis, polypeptides were blotted from gels onto nitrocellulose paper (0.45-μm pore size) using an electroblot technique modified from Burnette (2). Prior to electroblotting, a gel "sandwich" was prepared by placing the gel onto a sheet of nitrocellulose paper and backing this with sheets of DNA blotting paper (BRL). All materials were thoroughly wetted with Burnette's (2) electroblot transfer buffer (2 mM Tris-base, 192 mM glycine, and 20% [v/v] methanol) before "sandwich" construction. Electroblots were conducted at 20°C in a TE 42 Transphor electrophoretic transfer unit (Hoefer), filled with transfer buffer, with current at 0.2 A for 12–16 h. The system was cooled to 20°C via water circulation through an immersible glass core. Blots were removed from the "sandwich" after transfer and either used immediately or stored dry at −20°C.

Preparation of antisera to exospore proteins. Balb-C mice (Charles River Laboratories) were employed for the production of antisera to intact spores of *Nosema locustae, N. heliothidis, Vairimorpha necatrix, V. heterosporum,* and *V. plodiae.* Mouse antiserum to spores of *Encephalitozoon cuniculi,* a microsporidium of mammals, was provided by J. Shadduck, College of Veterinary Medicine, University of Illinois at Urbana-Champaign.

Spore samples, purified as described above, were removed from storage in DW at 4°C and washed twice with phosphate-buffered saline (PBS), pH 7.3. For each antigen preparation, ca. 5×10^8 spores were suspended in 1 ml of PBS and mixed thoroughly with 1 ml of Freund's complete adjuvant (FCA). Ten-week-old mice received intraperitoneal injections of 0.2 ml of the spore preparation (ca. 5×10^7 spores), followed by five similar injections at two-week intervals. The injection schedule employed is similar to those utilized for the production of hybridomas but is unusual for producing antisera in that the injections were all intraperitoneal and FCA was inadvertently utilized throughout the course of immunization. Normally, only the primary injection utilizes FCA and in all subsequent injections Freund's incomplete adjuvant is mixed with antigen.

Mice were bled from the retro-orbital plexus of the eye one week after the third injection and every other week thereafter for three additional bleedings. Blood was centrifuged at 15,600 *g* for 2 min and serum removed to a clean tube and stored at −20°C until needed.

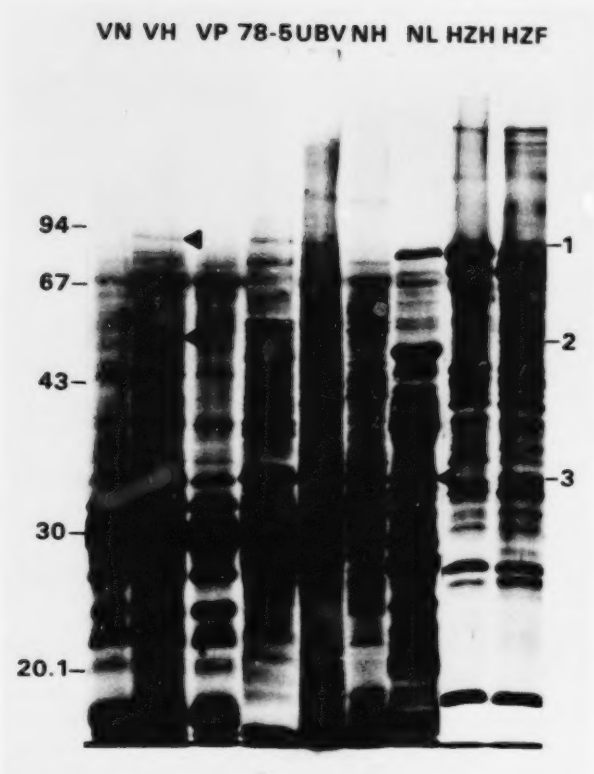

Fig. 1. SDS-PAGE of exospore and host polypeptides with silver staining. Abbreviations are *Vairimorpha necatrix* (VN), *V. heterosporum* (VH), *V. plodiae* (VP), undescribed *Vairimorpha*-like isolates (78-5, UBV), *Nosema heliothidis* (NH), *N. locustae* (NL), *Heliothis zea* hemolymph (HZH), and *H. zea* fat body (HZF). Positions of molecular weight marker proteins are shown on the left. Numbers given are actual molecular weight $\times 10^{-3}$. Numbers (corresponding to arrows) on right are: 1 and 2, unique bands of VH; 3, comigrating bands of NH and NL.

Control sera were obtained from 18 naive mice from the same shipment as immunized mice. Serum samples from the control mice were pooled and stored after separation from blood as described above.

Immunoblot assay procedure. Gel replicas on nitrocellulose sheets were used in an immunoblot assay (18, as modified by 20, 28) employing mouse anti-microsporidian spore sera and peroxidase-conjugated goat anti-mouse IgG (Sigma) second antibody. Assays were carried out in 13 × 7 × 6-cm styrene acrylonitrile utility boxes (Nalgene) and with shaking at room temperature. Nitrocellulose sheets were first incubated in 10 ml of NET/NP-40/AHS buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM Na-EDTA, 0.25% swine skin gelatin [w/v], 0.05% Nonidet NP-40 [v/v], 0.01% thimerosal [w/v], and 2% agamma horse serum [v/v]), pH 7.4, for 30 min. The buffer was decanted and 20 ml of fresh buffer were added, followed by 40 μl of antiserum or control sera. After overnight incubation, nitrocellulose sheets were washed for 5 min with 10 ml of NET/NP-40/AHS buffer. The solution was removed; 10 ml of fresh buffer and 20 μl of peroxidase-conjugated goat anti-mouse IgG were added and incubated for 2 h. Sheets were then washed with 10 ml of PBS-0.05% Non-idet NP-40, followed by two 5-min washes with 10 ml of PBS-10 mM EDTA. After the final wash, 10 ml of freshly made peroxidase substrate (0.03% [w/v] 3,3′-diaminobenzidine dissolved in sufficient 1% 1 N HCl, and 0.005% [v/v] hydrogen peroxide in a 10 mM EDTA, 50 mM Tris-HCl buffer, pH 7.5) were added and incubated for 10–20 min. Sheets were rinsed twice with DW and then dried between DNA blotting pads (BRL), where they were subsequently stored. All immunoblot assay sheets were photographed for analysis.

Immunoferritin localization of spore protein. An indirect immunoferritin procedure was utilized to determine whether spore proteins reacting with mouse anti-*V. necatrix* antisera (VNA) were surface components. Purified *V. necatrix* spores were fixed overnight at 4°C in 1% formaldehyde (prepared fresh from paraformaldehyde [16]) in 10 mM sodium phosphate buffer (PB), pH 7.2. Spores were washed four or five times (5 min each with shaking) by repeated suspension in PBS followed by centrifugation. The spores were then aliquoted into four 1.5-ml polypropylene centrifuge tubes and pelleted. All incubations and rinses were carried out on a shaker at room temperature. The general indirect immunoferritin procedure utilized spores as follows: 1) incubation for 3 h with primary antibody (25 μl VNA) in 0.5 ml PBS containing 4% agamma horse serum (GIBCO) and 0.01% thimerosal (PBS-AT), 2) rinsing for 5 min each in PBS-AT, PB, and PBS-AT, 3) 3 h incubation with 25 μl ferritin-labeled goat anti-mouse IgG antibody (Sigma) in 0.5 ml PBS-AT, 4) rinsing four times for 15 min with PB. One aliquot of spores was exposed to the complete regimen described above. The other three served as controls. Two spore samples were exposed to normal mouse serum in place of VNA. One of these was incubated further with ferritin-labeled second antibody and the other not. The third control was incubated with VNA but ferritin-labeled goat anti-mouse IgG antibodies were omitted.

Preparation of these spores for electron microscopic examination was carried out as follows: Spores were fixed with 1% (v/v) glutaraldehyde in 60 mM PB for 45 min, washed in buffer three times for 15 min and then post-fixed in 1% (w/v) OsO_4 in 90 mM PB, pH 7.0, for 1 h. After several rinses in DW, the spores were suspended in 0.5% agar, dehydrated in ethanol followed by propylene oxide, and embedded in Spurr's epoxy resin. Sections were cut with a Reichert OMU-3 microtome and examined with a Philips EM-300.

RESULTS

SDS-PAGE of exospore polypeptides. The SDS-PAGE of polypeptides extracted from intact spores, followed by silver staining, revealed 15 or more bands for each of the microsporidia examined (Fig. 1). In general, 10 or more major bands were observed. Each of the three *Vairimorpha* species produced approximately 30 bands, with ca. 10 major and ca. 20 minor bands. The UBV isolate produced ca. 15 major bands and ca. 35 minor bands. This higher number of bands may represent internal spore proteins resulting from spore germination during sample preparation. This presumption is supported by SDS-PAGE of intentionally germinated spores of this and the other three *Vairimorpha* species (unpubl. observ.). A similar number of exospore polypeptides were seen for ungerminated spores of UBV (not shown) and the 78-5 isolate (Fig. 1). *Nosema heliothidis* produced 9–10 major and 20–25 minor bands while *N. locustae* produced ca. 20 major and 10–15 minor bands.

Coelectrophoresis of molecular weight (MW) markers showed that most of the extractable polypeptides had a relative molecular mass (MW) of less than 90,000 although most isolates produced one or more faint band of greater MW. The lower limit of resolution of the gel system was about 15,000 MW. Smaller polypeptides comigrated with the dye front.

Coelectrophoresis of *H. zea* fat body and hemolymph extracts

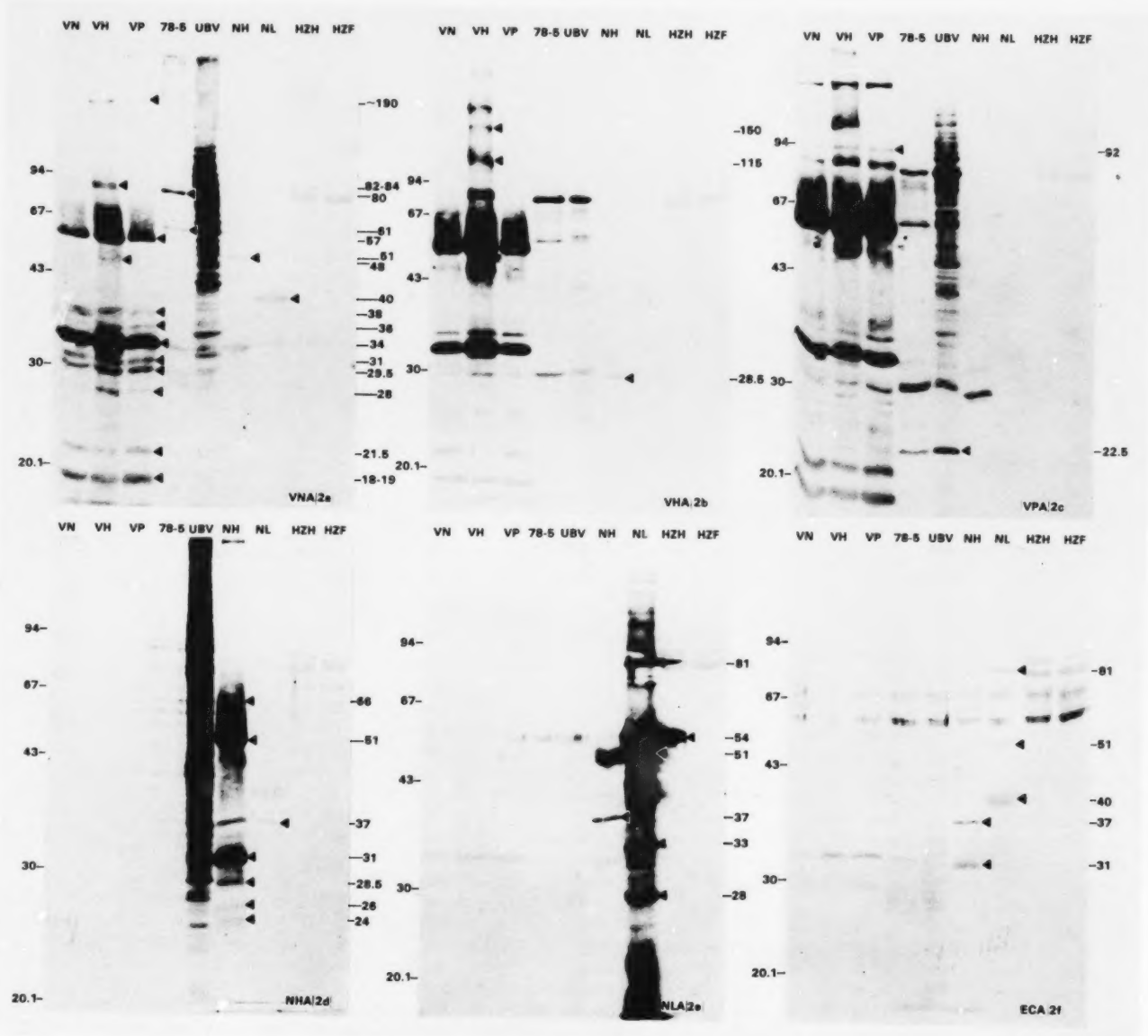

Fig. 2. Blots, proceeding from left to right, of polypeptides from *Vairimorpha necatrix* (VN), *V. heterosporum* (VH), *V. plodiae* (VP), undescribed *Vairimorpha*-like isolates (78-5, UBV), *Nosema heliothidis* (NH), *N. locustae* (NL), *Heliothis zea* hemolymph (HZH), and *H. zea* fat body (HZF) reacted with antisera produced against a) *V. necatrix* (VNA), b) *V. heterosporum* (VHA), c) *V. plodiae* (VPA), d) *N. heliothidis* (NHA), e) *N. locustae* (NLA), and f) *Encephalitozoon cuniculi* (ECA). Positions of molecular weight marker proteins are shown on the left. Molecular weights of polypeptide bands of interest (arrows) are shown on the right. Numbers shown are actual molecular weight $\times 10^{-3}$.

revealed minimal numbers of comigrating host and microsporidian polypeptides. Some of the *N. locustae* spore bands may represent host polypeptides because host (*Melanoplus* spp.) extracts were not available as controls.

Examination of gel banding patterns revealed four basic polypeptide patterns. They were 1) the *Vairimorpha* species pattern; 2) the 78-5 and UBV patterns; 3) the *N. heliothidis* pattern; and 4) the *N. locustae* pattern. The patterns of the three *Vairimorpha* species are essentially identical though *V. heterosporum* has two minor bands (Fig. 1, bands 1 and 2) not in those of *V. necatrix* and *V. plodiae*. The UBV and 78-5 patterns, while containing a few bands that also comigrate with those of the *Vairimorpha* species, share many comigrating bands. Each also possesses several unique bands. The patterns of *N. heliothidis* and *N. locustae* spores appear to be virtually unique. Only a single comigrating band (Fig. 1, band 3) is present in these two patterns.

Immunoblot assay of exospore polypeptides. Blot transfer of electrophoretically separated exospore polypeptides to nitrocellulose sheets followed by an enzyme-linked immunosorbent assay with mouse antisera against intact spore proteins allowed

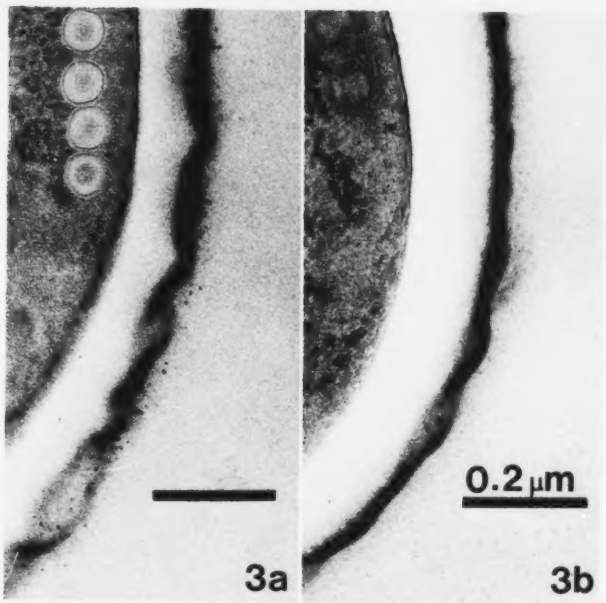

Fig. 3. Indirect immunoferritin demonstration of the occurrence of antigen(s) on the surface of *Vairimorpha necatrix* spores. Spores incubated with either mouse antiserum to *V. necatrix* spores (a) or control serum (b) were subsequently reacted with ferritin-conjugated goat antimouse-IgG antibodies. Ferritin deposition in (a) but not (b) specifically localizes one or more of the antigens on the spore coat surface.

visualization of homologous and cross-reacting polypeptides of seven microsporidia (Fig. 2). Blots were reacted with antisera to *V. necatrix* (VNA, Fig. 2a), *V. heterosporum* (VHA, Fig. 2b), *V. plodiae* (VPA, Fig. 2c), *N. heliothidis* (NHA, Fig. 2d), *N. locustae* (NLA, Fig. 2e), and *Encephalitozoon cuniculi* (ECA, Fig. 2f) as well as a control serum (not shown). Molecular weights of blot polypeptides were determined. A summary of the number of homologous and heterologous cross-reacting bands is given in Table II.

Antisera to *V. necatrix* (VNA), *V. heterosporum* (VHA), and *V. plodiae* (VPA) reacted similarly, though not identically, with blots (Fig. 2a–c). These antisera all reacted with major homologous antigens of 18–19,000, 21,500, 29,500, 34,000, 36,000, 57,000, and ca. 190,000 MW. Strongest reactions occurred with the 34,000 and 57,000 MW polypeptides on blots of all three *Vairimorpha* species. Notably, neither of these polypeptides are particularly prominent on silver-stained gels. Also, each antiserum exhibited unique reactivity with polypeptides possessed by all three *Vairimorpha* species. Antiserum to *V. necatrix* (VNA) (Fig. 2a) differed from the other antisera in that it reacted with polypeptides of 28,000 and 31,000 MW while VHA (Fig. 2b) revealed a minor polypeptide of 48,000 MW in all three species though this latter reaction was most prominent in the *V. heterosporum* blot. Notably, VNA and VPA revealed this polypeptide only in the *V. heterosporum* blot. Antiserum to *V. plodiae* (VPA) (Fig. 2c) was different from the previous two antisera in that it revealed polypeptides of 82–84,000 and 92,000 MW in the three *Vairimorpha* species; however, neither VNA nor VHA reacted with the 92,000-MW polypeptide, and the 82–84,000-MW doublet is seen only in the *V. heterosporum* blot when reacted with VNA or VHA. Finally, two polypeptides, of 115,000 and 150,000 MW, were revealed only on the *V. heterosporum* blots (Fig. 2b, c). Reaction to VHA occurred with both these polypeptides while VPA revealed only the 115,000-MW polypeptide.

Reactions of VNA, VHA, and VPA with blots of 78-5 and UBV (Fig. 2a–c) were similar though differences are apparent. Each antiserum reacted with polypeptides of 29,500, 61,000, and 80,000 MW present in blots of both microsporidia; VPA reacted uniquely with a 22,500-MW polypeptide (Fig. 2c). Finally, both VNA and VPA reacted with a wide array of presumably internal proteins present in the UBV blot while VHA did not exhibit such reactivity.

Very limited cross-reactions occurred between VNA, VHA, or VPA and blots of *Nosema* species. Antiserum to *V. necatrix* (VNA) reacted only with a 51,000-MW polypeptide from *N. heliothidis* and a 40,000-MW polypeptide from *N. locustae* (Fig. 2a) while VHA and VPA reacted only with a 28,500-MW polypeptide from *N. heliothidis* (Fig. 2b, c).

Antiserum to *N. heliothidis* (NHA) (Fig. 2d) reacted strongly with major homologous polypeptides with a 31,000 MW and with antigens in the 51,000- to 66,000-MW range. Weaker reactions occurred with MWs of 37,000, 28,500, 26,000, and 24,000. No cross-reactivity is visible with blots of the three *Vairimorpha* species or 78-5. The UBV blot, however, as with VNA and VPA, cross-reacted extensively with NHA. Cross-reactivity with the *N. locustae* blot was limited to a 37,000-MW polypeptide.

Antiserum to *N. locustae* (NLA) (Fig. 2e) reacted with many homologous polypeptides but major reactivity occurred with antigens of MW 81,000, 54,000, 33,000, and 28,000. The polypeptides in the MW range 37,000 to 54,000 stained so intensely that individual bands in this region could not be distinguished. Very weak, probably non-specific, cross-reaction of NLA with the 34,000-MW polypeptides of the *Vairimorpha* species blots occurred. Two major polypeptides of MW 51,000 and 37,000 on the *N. heliothidis* blots cross-reacted with NLA.

Antiserum to *Encephalitozoon cuniculi* (ECA) (Fig. 2f), a microsporidium of mammals, cross-reacted minimally with blots

TABLE II. *Common antigenic determinants of microsporidian spore polypeptides.*

Antiserum	NH[a]	NL	VH	VN	VP	UBV	78-5	HZH	HZF
NHA[b]	ca. 9	3	–	–	–	ca. 35	–	–	–
NLA	3	ca. 10	–	–	–	–	–	–	–
VHA	1	–	14–15	9–10	9–10	5	6	–	–
VNA	2	2	14	9	10	ca. 30	3	–	–
VPA	2	–	15–16	14–15	14–15	ca. 30	7–8	–	–
ECA	3	3	1	1	1	–	–	–	–
Control serum	–	–	–	–	–	–	–	–	–

[a] NH = *Nosema heliorhidis*; NL = *N. locustae*; VH = *Vairimorpha heterosporum*; VN = *V. necatrix*; VP = *V. plodiae*; UBV and 78-5 are undescribed isolate designations; HZH = *Heliothis zea* hemolymph; HZF = *H. zea* fat body.

[b] Mouse antiserum produced against microsporidian species, abbreviated as in [a] above; ECA is antiserum to *Encephalitozoon cuniculi*.

of entomophilic microsporidia. Polypeptides revealed include the 34,000-MW polypeptide of the three *Vairimorpha* species and 78-5. Staining of polypeptides with MW of 31,000, 37,000, and 51,000 occurred on the *N. heliothidis* blot. Reactions also occurred between ECA and *N. locustae* polypeptides of ca. MW of 40,000, 51,000, and 81,000. Apparent artifactual staining, of unknown origin, is seen in the 60–70,000 MW portion of the blot.

Control sera (not shown) showed no reactivity with blots.

Indirect immunoferritin localization of surface antigens. An indirect immunoferritin procedure permitted localization by transmission electron microscopy of immunoreactive sites on the exospore surface of *V. necatrix* spores. Examination of thin sections of whole *V. necatrix* spores incubated with VNA followed by ferritin-conjugated goat anti-mouse IgG revealed ferritin deposits on the exospore surface (Fig. 3a). When control serum was substituted for VNA or when the ferritin-conjugated second antibody was employed alone, ferritin deposition on *V. necatrix* exospores was eliminated (Fig. 3b).

DISCUSSION

Constituent polypeptides of the exospore proteins of a variety of microsporidian isolates were investigated by SDS-PAGE followed by silver nitrate staining or immunoblotting. The results indicate that different microsporidia possess a number of polypeptides that are both extractable and antigenic in the intact spores. The specificity or limited cross-reactivity of certain polypeptides in the immunoblot assay suggests their potential immunodiagnostic value. By development of monoclonal antibodies to these polypeptides, simpler assays may be employed. Earlier studies have reported the adaptation of such assays for use with entomophilic microsporidia, including microtitration plate enzyme-linked immunosorbent assays (ELISA) (8; unpubl. observ.), indirect fluorescent antibody tests (14, 19), and a spore agglutination assay (25).

It was presumed throughout our investigation that the majority of polypeptides resolved by SDS-PAGE and visualized via the immunoblot technique were obtained from the proteinaceous (13, 26, 27) exospore while a more recent investigation suggested the contrary (21). In the latter report, electrophoretically resolvable polypeptides from the exospores of two entomophilic microsporidia, including one species (*V. necatrix*) reported on herein, were not observable by Coomassie brilliant blue R-250 staining. It was suggested that either the extraction buffer system employed was insufficient or the presence of a richly invested proteinaceous exospore is not a generic feature of the microsporidia tested. Subsequent investigations by one of the authors (D. A. Streett, pers. commun.) indicated that the extraction buffer system was indeed insufficient and required a minimum of 2% SDS for successful extraction of polypeptides from intact spores.

Several other lines of evidence produced in the present investigation further confirm our presumption that many of the polypeptides visualized by SDS-PAGE and the immunoblot assay are from the exospore. First, examination of spores by phase-contrast optics before and after polypeptide extraction revealed no change in birefringence of the spore interior. This indicates that neither extensive spore germination nor extraction of internal components occurred. This observation, however, does not preclude the possibility of small amounts of polypeptide "seepage" from the interior since spore permeability, particularly to cations and anions, has been documented for a variety of entomophilic microsporidia (23). Second, when electrophoretograms of polypeptides extracted from intact spores and from germinated spore preparations (containing both empty spores and the extruded sporoplasms) of the same isolate were prepared, marked enhancement of those bands produced from intact spore extracts alone was not observed (unpubl. observ.). Furthermore, the absence in silver-stained gels and blots of polar filament protein, consisting of monomeric polypeptides of 23,000 MW (29), indicates that extensive germination and extraction of internal polypeptides did not occur. Finally, localization of surface antigens by the indirect immunoferritin technique demonstrates that some, if not all, of the antigenic epitopes are present on the surface of the exospore. The present evidence suggests strongly that exospore proteins are sufficiently diverse to provide a biochemical and immunological basis for the identification of microsporidia and for the assessment of evolutionary relationships among species and genera.

While our investigation was not primarily taxonomic, we have shown that significant structural divergences of taxonomic import occur in the exospore proteins of entomophilic microsporidia. The genus *Vairimorpha*, erected as separate from *Nosema* (17) after the discovery of temperature-dependent dimorphic development in *Vairimorpha* (*Nosema*) *necatrix* (6) has been shown by immunoblot assays to share a minimal serological relationship with at least two members of the genus *Nosema*. Such serological divergence has previously been demonstrated for these two genera by double immunodiffusion assays (11). The extensive cross-reactivity of NHA, VNA, and VPA with UBV blots (Fig. 2a, c, d), which contain a high number of internal proteins, suggests, however, that many such proteins may be held in common among members of the genera *Nosema* and *Vairimorpha*. The presence of antibodies to internal proteins may be due to spore germination in experimental animals as has been demonstrated (24). Further studies should clarify this issue.

The three described *Vairimorpha* species, *V. necatrix, V. heterosporum,* and *V. plodiae* are almost indistinguishable by the techniques we employed, with only minor differences apparent. These results concur with earlier electrophoretic studies, which showed *V. necatrix* and *V. plodiae* to be indistinguishable (5) while *V. heterosporum* was shown to be only slightly less closely related (21). We found these three *Vairimorpha* species to be virtually indistinguishable by SDS-PAGE; only when blots were reacted with VNA, VHA, and VPA, were differences in immunoreactivity apparent. By all conventional immunotaxonomic criteria these *Vairimorpha* species would appear to be conspecific. Substantial further studies need to be conducted with a greater variety of geographic isolates of these species in order to gain a fuller understanding of this complex.

The two undescribed isolates examined in this investigation have been shown to possess *Vairimorpha*-like dimorphism (UBV: S. Jaronski; 78-5: J. J. Hamm, pers. commun.); however, the results of the immunoblots (Fig. 2a–c) suggest that these isolates, while related to the *Vairimorpha* species, are not closely related. Aside from the above exception with NHA and UBV blots, they show no relation to the *Nosema* species. They do appear, however, by both SDS-PAGE and the immunoblot assay, to be related to each other.

Nosema locustae and *N. heliothidis* are readily distinguished from each other and from the other microsporidia by both the immunoblot assay and SDS-PAGE. In fact, the immunoblot assay (Fig. 2; see also Table II) shows them to be only slightly more related to each other than to the *Vairimorpha* species. This relative unrelatedness is illustrative of the heterogeneous nature of the genus *Nosema* noted by a number of authorities on Microspora. The cross-reactions between the two *Nosema* blots and ECA may be additional evidence of this heterogeneity.

Of primary importance in our investigation was the ability to distinguish between *N. locustae* and other microsporidia. Within the limited number of isolates we examined, *N. locustae*

spores were sufficiently immunologically unique to encourage further investigation via monoclonal antibody techniques. Inclusion of other orthopteran microsporidia, such as *N. acridophagus* and *N. cuneatum*, in further studies of this nature should help clarify the status of *N. locustae* as immunologically distinct.

LITERATURE CITED

1. Brooks, W. M. 1985. Protozoa, *in* Ignoffo, C. M., ed., *Microbial Insecticides*, Vol. 5, *Handbook of Naturally Occurring Pesticides*. CRC Press, Boca Raton, Florida. (in press)
2. Burnette, W. N. 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.*, **112**: 195–200.
3. Conlon, J. M. 1980. *Nosema locustae*: exemption from requirement of a tolerance. *Fed. Register*, **45**: 31312–31313.
4. Fowler, J. L. & Reeves, E. L. 1974a. Detection of relationships among microsporidian isolates by electrophoretic analysis: hydrophobic extracts. *J. Invertebr. Pathol.*, **23**: 3–12.
5. ——— 1974b. Detection of relationships among microsporidian isolates by electrophoretic analysis: hydrophilic extracts. *J. Invertebr. Pathol.*, **23**: 63–69.
6. ——— 1974c. Spore dimorphism in a microsporidian isolate. *J. Protozool.*, **21**: 538–542.
7. Gershoni, J. M. & Palade, G. E. 1983. Protein blotting: principles and application. *Anal. Biochem.*, **131**: 1–15.
8. Greenstone, M. H. 1983. An enzyme-linked immunosorbent assay for the *Amblyospora* sp. of *Culex salinarius* (Microspora: Amblyosporidae). *J. Invertebr. Pathol.*, **41**: 250–255.
9. Hazard, E. I., Ellis, E. A. & Joslyn, D. J. 1981. Identification of Microsporidia, *in* Burges, H. D., ed., *Microbial Control of Pests and Plant Diseases, 1970–1980*. Academic Press, New York, pp. 163–182.
10. Henry, J. E. & Oma, E. A. 1981. Pest control by *Nosema locustae*, a pathogen of grasshoppers and crickets, *in* Burges, H. D., ed., *Microbial Control of Pests and Plant Diseases, 1970–1980*. Academic Press, New York, pp. 573–586.
11. Knell, J. D. & Zam, S. G. 1978. A serological comparison of some species of microsporidia. *J. Invertebr. Pathol.*, **31**: 280–288.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, **227**: 680–685.
13. Maurand, J. & Loubès, C. 1973. Recherches cytochimiques sur quelques microsporidies. *Bull. Soc. Zool. Fr.*, **98**: 373–383.
14. Niederkorn, J. Y., Shadduck, J. A. & Weidner, E. 1980. Antigenic cross-reactivity among different microsporidian spores as determined by immunofluorescence. *J. Parasitol.*, **66**: 675–677.
15. Oakley, B. R., Kirsch, D. R. & Morris, N. R. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.*, **105**: 361–363.
16. Pease, D. C. 1964. *Histological Techniques for Electron Microscopy*. Academic Press, New York, 381 pp.
17. Pilley, B. M. 1976. A new genus, *Vairimorpha* (Protozoa: Microsporidia), for *Nosema necatrix* Kramer 1965: pathogenicity and life cycle in *Spodoptera exempta* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol.*, **28**: 177–184.
18. Renart, J., Reiser, J. & Stark, G. R. 1979. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc. Natl. Acad. Sci. USA*, **76**: 3116–3120.
19. Sato, R., Kobayashi, M., Watanabe, H. & Fujiwara, T. 1981. Serological discrimination of several kinds of microsporidian spores isolated from the silkworm, *Bombyx mori*, by an indirect fluorescent antibody technique. *J. Seric. Sci. Jpn.*, **50**: 180–184. (in Japanese with English summary)
20. Smith, G. & Summers, M. D. 1981. Application of a novel radioimmunoassay to identify baculovirus structural proteins that share interspecies antigenic determinants. *J. Virol.*, **39**: 125–137.
21. Streett, D. A. & Briggs, J. D. 1982. An evaluation of sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the identification of Microsporidia. *J. Invertebr. Pathol.*, **40**: 159–165.
22. ——— 1983. Variation in spore polypeptides from four species of *Vairimorpha*. *Biol. Syst. & Ecol.*, **10**: 161–165.
23. Undeen, A. H. 1978. Spore hatching processes in some *Nosema* species with particular reference to *N. algerae*, Vávra and Undeen. *Misc. Publ. Entomol. Soc. Am.*, **11**: 29–49.
24. Undeen, A. H. & Alger, N. E. 1976. *Nosema algerae*: infection of the white mouse by a mosquito parasite. *Exp. Parasitol.*, **40**: 86–88.
25. ——— 1977. Agglutination and immunofluorescent tests for infection of mammals by *Nosema algerae* (Cnidospora: Microsporida). *Sci. Biol. J.*, **3**: 259–262.
26. Vávra, J. 1976. Structure of the Microsporidia, *in* Bulla, L. A., Jr. & Cheng, T. C., eds., *Comparative Pathobiology, Biology of the Microsporidia*. Plenum Press, New York **1**: 1–85.
27. Vivarès, C.-P., Loubès, C. & Bouix, G. 1976. Further cytochemical researches on microsporidian parasites of the Mediterranean shore crab, *Carcinus mediterraneus* Czerniavsky, 1884. *Ann. Parasitol. Hum. Comp.*, **51**: 1–14.
28. Volkman, L. E. 1983. Occluded and budded *Autographica californica* nuclear polyhedrosis virus: immunological relatedness of structural proteins. *J. Virol.*, **46**: 221–229.
29. Weidner, E. 1976. The microsporidian invasion tube. I. The structure, isolation, and characterization of the protein comprising the tube. *J. Cell Biol.*, **71**: 23–34.

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